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# Mycobactins: Iron-Chelating Growth Factors from Mycobacteria

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## DISCOVERY AND EARLY HISTORY OF MYCOBACTINS

Mycobacteria were early recognized to form a compact group of organisms, linked in the first place by their property of "acid-fastness." Among these organisms were various pathogens and some saprophytes. Most of these species of mycobacteria could be grown on laboratory media, but two could not. One was the bacillus found in the lesions of leprosy, which still presents great difficulties in culture. The other was the organism characteristic of the condition of chronic enteritis in cattle known as Johne's disease and called *Mycobacterium paratuberculosis*. The old name of *M. johnei*, now disallowed, is still used by some authors who may also refer to it as Johne's bacillus. Twort and Ingram (85, 86) attempted unsuccessfully to grow *M. paratuberculosis* by supplementing the medium with extracts of cattle tissues. They postulated that failure to cultivate the organism "must be due to the absence of some necessary foodstuff" and that the missing substance would most likely be found in tubercle bacilli. They showed that when dried, killed, human tubercle bacilli were added to their egg medium a good growth of *M. paratuberculosis* developed. They were also able to obtain growth with other mycobacteria and with extracts prepared from them, using organic solvents. Twort concluded, very acutely, that all the organisms in this group of mycobacteria needed an "essential substance" that was vital to their growth. Most mycobacteria were able to make this substance for themselves, but *M. paratuberculosis* lacked this capacity. He assumed that this growth substance was present in tubercle bacilli even when "no reserve was formed that can be extracted." Twort's findings and deductions have been amply confirmed and it is evident that he had a clear concept, even in 1911 when his findings were first reported, of the role of essential metabolites and growth factors. In this he must be reckoned a true pioneer since ideas of vitamins and growth factors were only dimly recognized at that time. Since Twort's discovery, simple extracts of *M. phlei* have been used regularly to supplement the media used in the culture of *M. paratuberculosis*, but no actual attempt to isolate the growth factor was made for more than 30 years.

The potential chemotherapeutic significance of Twort's observations was first pointed out in 1945 by J. Francis, working in the laboratories of Imperial Chemical Industries, Ltd. At this time, no chemotherapeutic treatment was available for tuberculosis. Francis argued that since a growth factor for *M. paratuberculosis* was produced by *M. tuberculosis* and seemed highly specific for the mycobacteria, its structure, if known, should provide a highly suitable model for the synthesis of compounds having a specific action against the mycobacteria. Since animals and man did not make or depend on the growth factor, an antagonist should not be toxic to them, nor should its action be swamped by an excess of growth factor from the host's tissues. Thus the *M. paratuberculosis* growth factor promised to be a much more suitable model for the design of an antagonist than the more ubiquitous growth factors then known. The first necessity was the isolation of the growth factor in a pure form. For this purpose, the nonpathogenic and easily grown organism *M. phlei* was chosen. This proved a fortunate choice in several ways. On suitable media it produces relatively large amounts of growth factor; it also happens to be the only organism from which a crystalline product with growth-promoting action for *M. paratuberculosis* has yet been obtained. The extraction and concentration of the growth factor from large quantities of *M. phlei* was undertaken by J. Madinaveitia and H. M. Macturk. Their work was hampered by the inaccurate and temperamental assay then available. The isolation of the pure growth factor was greatly helped however by the crystallization of the aluminum complex (28) which was formed adventitiously in attempts to separate the factor on a column of chromatographic alumina. X-ray crystallography of the thin, fragile, monoclinic crystals gave the dimensions of the unit cell and established the molecular weight as 1,828 $\pm$ 73 per unit cell. It was clear from the elementary analysis that the unit cell must contain two molecules of the complex and that each molecule contained a single aluminum atom. This meant that the growth factor probably had a large and complex structure. Early attempts to obtain the metal-free growth factor from the aluminum complex were unsuccessful, though with later knowledge it can now be done easily. A different purification procedure eventually led to the isolation of the metal-free growth factor (27) and to the establishment of its principal chemical properties; it was named mycobactin. Degradation studies (69, 70) revealed the main features of the chemi-

cal structure, though some uncertainties remained. The complete structure, including the stereochemistry of the five asymmetric centers, was settled later (71). The stereochemistry of the groups around the metal atom in the aluminum complex and the analogous ferric complex is still undetermined but should be settled by X-ray crystallography now in progress.

The successful extraction of mycobactin from *M. phlei* was followed by attempts to obtain the growth factor from *M. tuberculosis*. This proved much more difficult. It was necessary to use a medium on which the organism grew rather slowly and sparsely, and the yield of growth factor per unit weight of cells was much lower than with *M. phlei*. Moreover, the method used for isolating the mycobactin from *M. phlei* entirely failed with *M. tuberculosis*. In *M. phlei*, the mycobactin was present mainly in a metal-free form, but in *M. tuberculosis* it was linked to iron and therefore had quite different physical properties. An altered extraction method applied to *M. tuberculosis* gave a mycobactin which differed structurally in several ways from the mycobactin derived from *M. phlei* (72). The mycobactins were distinguished by the letters T and P; all earlier work with mycobactin should now be regarded as referring to mycobactin P. The system of lettering has since been extended to cover other mycobactins isolated from various mycobacterial species. The isolation of mycobactin T explained an earlier observation on mycobacterial growth factors. Marks (46) found that ether extracts of *M. tuberculosis* resembled mycobactin P in stimulating the growth of *M. tuberculosis* on an unfavorable alkaline serum medium. Mycobactin P could not be extracted from *M. phlei* with ether, and this suggested that the growth factor in *M. tuberculosis* might not be a mycobactin. However, it is now evident that the material extracted by Marks was the ether-soluble ferric complex of mycobactin T.

At the outset, a considerable search was made for growth factors for *M. paratuberculosis* in sources other than mycobacteria (27, 86, 87). Although extracts of various materials of plant and animal origin appeared to show some growth-promoting action, the effect was relatively feeble and difficult to reproduce. Williams-Smith (95) examined extracts of tissues, rumen contents, intestinal contents, and feedstuffs of cattle and other animals, some infected with Johne's disease; none of these extracts was able to stimulate the growth of *M. paratuberculosis*. The only established and consistent sources of growth factor for *M. paratuberculosis* are the various species of mycobacteria. This high degree of specificity within a bacterial group gives the mycobactins a unique position among growth factors.

The original intention to use the mycobactin structure as a model for drugs having a specific action against mycobacteria was frustrated by the complexity of the molecule. It proved difficult to make compounds sufficiently close in structure to the mycobactins to give hope of antagonistic action. The closest structural analogue yet synthesized is a relative of mycobactin T lacking the characteristic iron-chelating groups (22); this compound did not affect the growth of *M. tuberculosis*. Some growth antagonism effects have been obtained with chromic mycobactin P and with mixtures of mycobactins, though these have no practical therapeutic value. Among analogous iron-chelating growth factors from other microorganisms, effective naturally occurring antagonists have been found. Thus, the concept of a mycobactin antagonist seems to have been sound in principle, though not yet realized in practice.

The isolation of mycobactin was followed by a number of reports of bacterial growth factors, apparently dissimilar but linked by the common feature of a strong chelating ability for ferric iron. They included Terregens Factor (45), coprogen (36), ferrichrome (54), and the ferrioxamines (9). These compounds have some similarity to the mycobactins in possessing hydroxamic acid groups, but otherwise they are very different chemically; biologically their functions may show closer parallels. The naturally occurring iron-chelating hydroxamic acids have been given the generic name of "siderochromes." Within this class, the growth factors containing three hydroxamic acid groups are called "sideramines" and related compounds having a growth-antagonistic effect are named "sideromycins" (9). The implications of these terms and the relationships between mycobactins and sideramines are discussed later.

Two general reviews on siderochromes have appeared in recent years (40, 55). A thoughtful review by Hanks on host-dependent microorganisms (34) discusses the biological problems presented by the dependence of *M. paratuberculosis* on mycobactin.

## PRODUCTION, EXTRACTION, AND PURIFICATION OF MYCOBACTINS

### Conditions for Production of Mycobactins: Influence of Iron Deficiency

The influence of the composition of the medium used for the growth of mycobacteria on the amount of mycobactin produced was noticed during the original work on the isolation of my-

cobactin P (27). Although *M. phlei* could be readily grown on various synthetic media, the production of mycobactin P was negligible, and it was only by the use of beef infusion broth that substantial yields could be achieved. Later, the decisive factor was found to be the concentration of iron in the medium. As with other natural iron-chelating compounds, production is greatly stimulated by a deficiency of iron. The growth of mycobacteria is reduced at low iron concentrations. Turian (82) showed that addition of 0.04 μg of iron per ml increased the growth of *M. phlei* in a basal synthetic medium by 50%, and maximal growth was attained at 0.4 μg/ml.; the iron content of the basal medium was not determined, however. Antoine and Morrison (2) also grew *M. phlei* on a synthetic medium in which the measured iron content was 0.03 μg/ml. In this medium, growth became maximal in 7 to 9 days and then declined. At a concentration of 0.23 μg of Fe per ml, a similar growth rate was achieved at 7 days but continued for a much longer period; growth was not greatly increased by raising the iron concentration to 1.60 μg/ml. Measurements were also made of the hydroxylamine-N released on acid hydrolysis of methanolic extracts of the cells, and of the changes in iron content of the medium during growth. The hydroxylamine-N measurements were shown to reflect the production of mycobactin as measured by bioassays. With an initial iron concentration of 0.03 μg/ml, the production of hydroxylamine-N corresponded fairly closely to the growth of the cells, reaching a maximum at 10 to 12 days. With an initial iron concentration of 0.23 μg/ml, production of hydroxylamine-N was very slight during the first 12 days. During this time the concentration of iron in the medium declined; production of hydroxylamine-N began when a very low iron level was reached and then rose sharply, but did not reach as high a level as in the experiment with the lower initial level of iron. In both experiments the concentration of hydroxylamine-N reached a maximum and then declined. When the medium contained initially 1.60 μg of Fe per ml, little production of hydroxylamine-N occurred. The iron content of the medium dropped steadily during the whole growth period, but even after 19 days it was still too high to allow much mycobactin production. Further information has been provided by Winder and O'Hara (96) who measured the iron content of cells of *M. smegmatis* grown under conditions of iron limitation. These results demonstrate quantitatively what had been found empirically in growing mycobacteria for the production of mycobactins. It is necessary to use a medium with a sufficiently low iron content and to experiment with different growth times to get the maximal yield of mycobactin. Iron concentration is difficult to measure and to control at these low levels. Fortunately, for practical purposes a synthetic medium prepared from ordinary analytical reagent chemicals contains less than 0.15 μg of iron per ml (93); the actual content is usually about 0.10 μg/ml. This is sufficient to support reasonable growth of mycobacteria and to give good yields of the mycobactins. Under these conditions, the mycobactins are produced mainly in the metal-free form; any remaining iron in the medium is quickly taken up as the stable ferric complex. If the amount of mycobactin production is very low, as with *M. tuberculosis*, the mycobactin may be found largely in the ferric form (72). Table 1 shows the composition of suitable synthetic media (93). For many mycobacteria, medium I containing 5% glucose and 1% glycerol is satisfactory, but for *M. tuberculosis* and *M. terrae* medium II containing less glucose and more glycerol is preferable. The growth temperature should be chosen to suit the particular strain of mycobacterium. In these laboratories, mycobacteria for mycobactin production have been grown on the surface of liquid medium, the growth period being either 2 or 3 weeks according to the organism. At least some of the mycobacteria can be grown in stirred or shaken cultures and will produce mycobactins under these conditions. The growth of organisms is then considerably quicker; the use of steel in vessels or stirrers must be avoided.

### Extraction Methods

The mycobactins are associated with the mycobacterial cells, although it is not established whether they are inherently intracellular or remain associated with the cells because of their low solubility in water. The cells can be removed from

TABLE 1. *Compositions of media for the growth of mycobacteria for mycobactin production*

| Solution[a] | Medium 1 | Medium 2 |
|---|---|---|
| First solution | | |
| $KH_2PO_4$ A.R. | 1 g | 1 g |
| $Na_2HPO_4$ A.R. | 2 g | 2 g |
| Glycerol A.R. | 10 ml | 30 ml |
| L-Asparagine | 5 g | 5 g |
| Deionized distilled water | 500 ml | 500 ml |
| Second solution | | |
| Glucose A.R. | 50 g | 10 g |
| $MgSO_4$, $7H_2O$ A.R. | 0.2 g | 0.2 g |
| Deionized distilled water | 500 ml | 500 ml |

[a] Solutions were separately autoclaved and mixed aseptically. A .R. indicates analytical reagent.

the medium by filtration or by centrifugation, and are washed free of medium. Various methods of extraction have been used, but the most convenient is to suspend the cells in cold ethanol for 1 day. This kills the bacteria and extracts the mycobactins, leaving much of the fatty material unextracted. The use of hot solvents extracts much unwanted fatty material and complicates the subsequent purification. The purification methods originally used for mycobactin P (27) were based on the following properties: (i) solubility of mycobactin P in cold ethanol and in chloroform and its insolubility in light petroleum or in ether; (ii) formation of a copper complex insoluble in ethanol; and (iii) formation of a crystalline aluminum complex. These methods have now been replaced by a general method applicable to all the mycobactins so far studied (74, 94). The extracted mycobactin, sometimes already partly in the ferric form, is completely converted to the ferric complex which is extracted with chloroform to give a solid product. Extraction with cold methanol removes the ferric complex, leaving some undissolved impurities. The crude product after evaporation of the methanol contains from 3 to 40% of ferric mycobactin according to the organism used. The yields of mycobactin also vary considerably with the strain. Figures of typical yields are shown in Table 2. These yields depend on the exact conditions of culture, but some types of mycobacteria certainly produce mycobactins much more readily than others. Our strain of *M. fortuitum* gave a mycobactin yield representing about 2% of the dry weight of the cells, whereas with *M. kansasii* the content was 0.05% or less.

Originally, concentration and purification of the mycobactins was controlled by biological assay. However, measurement of the characteristic extinction at 450 nm for the ferric complex dissolved in chloroform now provides an easier and more accurate guide. Correction can be made for extraneous absorption at the selected wavelength by subtracting the extinction measured after dissociating the ferric complex by shaking the chloroform solution with 5 M hydrochloric acid. This method was shown to give good agreement with the biological assay in fractions varying greatly in mycobactin content (91).

TABLE 2. *Yields of dried cells and ferric mycobactins from various mycobacteria grown on synthetic medium*[a]

| Organism | Dried cells | Ferric mycobactin | | | |
|---|---|---|---|---|---|
| | | Crude extract | Ferric mycobactin in extract | Amt/dried cells | Amt/medium |
| | g | mg | % | mg/g | mg/10 liters |
| *Mycobacterium phlei* | 10.8 | 350 | 31 | 10.1 | 110 |
| *M. tuberculosis* | 4.8 | 101 | 8.9 | 1.9 | 9 |
| *M. smegmatis* | 7.5 | 403 | 32 | 17.3 | 130 |
| *M. thermoresistible* | 7.1 | 390 | 21 | 11.8 | 84 |
| *M. terrae* | 15.2 | 90 | 38 | 2.3 | 35 |
| *M. fortuitum* | 11.5 | 660 | 34 | 19.5 | 224 |
| *M. aurum* | 10.1 | 180 | 4 | 0.7 | 7 |
| *M. kansasii* | 9.8 | 160 | 2.8 | 0.5 | 4 |
| *M. marinum* | 8.7 | 1170 | 2.5 | 3.4 | 30 |
| *M. balnei* | 8.0 | 270 | 16 | 5.4 | 43 |
| *M. piscium* | 9.4 | 460 | 15 | 7.3 | 69 |
| *M. thamnopheos* | 7.4 | 100 | 6.9 | 0.9 | 7 |

[a] Values refer to organisms from 10 liters of medium.

### Purification Procedures

The general method of purification of the ferric mycobactins after the preliminary extraction involves adsorption chromatography on alumina using cyclohexane–*tert*-butanol mixtures, sometimes with the addition of a little propylene glycol (94). The number of chromatographic steps required depends on the mycobactin and on the amount of impurity present in the crude extract. Three successive purification steps are usually sufficient to give the pure ferric complex, but sometimes a single step is enough. For bulk separations columns are used, but small quantities can be handled by preparative thin-layer chromatography. For mycobactin R and for the mycobactins from *M. marinum*, the chromatographic purification is replaced by counter-current separation using a quaternary system of cyclohexane, *tert*-butanol, propylene glycol, and water (74). Purity of the ferric complex is assessed by the attainment of a steady $E_{450}$ value which cannot be raised by further purification. The ferric complexes of the known mycobactins all have $E^{1\%}_{1cm}$ values at 450 nm lying between 40 and 42. With mycobactin P, the aluminum complex can be recrystallised to constant melting point as a further measure of purification, but the aluminum and ferric complexes of other mycobactins have not been crystallized.

The purified ferric mycobactin is converted to the metal-free mycobactin by shaking a chloroform solution repeatedly with 5 M hydrochloric acid. The washed and dried solution is evaporated, the residue is dissolved in a little dry chloroform, and the mycobactin is precipitated with dry ether. This precipitation step can be repeated if necessary and is useful in removing

residual impurities when complete purification has not been achieved at the ferric complex stage.

### Mycobactins as Mixtures of Homologous Compounds: Criteria of Purity

In speaking of the "purity" of ferric mycobactins and mycobactins, it must be recognized that these are not generally single chemical species, but mixtures of homologues having fatty side chains of various lengths; purity refers to the absence of extraneous impurities and absence of mycobactins having other structures in the nucleus of the molecule. The chemical structures of the different mycobactins are given later. Most mycobacteria produce one type of mycobactin in great preponderance; small amounts of other mycobactins may be detectable (94) but these are eliminated during the purification. Mycobactins A and R were apparently produced by our strains as single chemical species with only traces of homologous compounds, but all the other mycobactins were mixtures which were very difficult to separate. With mycobactin P, one component predominated and was freed from its homologues by counter-current distribution (93).

The presence and relative abundance of homologous compounds is shown by mass spectrometry of the aluminum complexes and by vapor phase chromatography of derivatives of the fatty acid fragments obtained by chemical degradation.

Two organisms were found that produced mixed mycobactins having different molecular nuclei. Our strain of *M. marinum* produced mycobactins M and N which were easily separated, whereas *M. fortuitum* produced mycobactins F and H which were inseparable; their presence was shown by mass spectrometry and by the results of chemical degradation of the mixture (74).

## PROMOTION OF GROWTH OF MICROORGANISMS BY MYCOBACTINS

### Assay Methods

The original method of assay of the mycobactins (27), which involved the growth of *M. paratuberculosus* on solid egg medium, was slow and troublesome. It has been gradually improved, principally by the introduction of Hart's liquid medium (35), and now provides a reliable method giving accurate results in 10 to 14 days. The main principles of the assay were established by Wheater and Snow (91), but later modifications (74) stressing the use of young cultures for inoculation and standarization of the inoculum by nephelometry have improved the method in reliability and accuracy. The *M. paratuberculosis* assay is highly specific for the mycobactins, but is still relatively slow. The use of *Arthrobacter terregens* provides an agar-plate diffusion method for rapid screening, or a tube test that can be completed in 3 days or less (3, 65). This organism is much less specific, giving a positive response with various sideramines, with some synthetic chelating agents and with cobactin and mycobactic acid, the primary breakdown products of mycobactin. However, a suitable extraction procedure could exclude many possible interfering substances since the solubility properties of the mycobactins are quite distinct from those of other sideramines and naturally occurring hydroxamic acids. The *M. paratuberculosis* test requires concentrations of mycobactin P between 5 and 15 ng/ml; the *A. terregens* test requiring between 100 and 250 ng/ml is considerably less sensitive, though adequate for many purposes. Both operate over a rather narrow concentration range but the more linear relationship between concentration and turbidity with *A. terregens* is advantageous.

### Growth Characteristics of M. paratuberculosis in the Presence of Different Mycobactins

Growth response in the *M. paratuberculosis* test is not entirely consistent from one experiment to another, and measurements should always be based on comparison with mycobactin solutions of known concentration. Provided that the test mycobactin is of the same type as the control, this presents no problems but different mycobactins may give different shapes of growth curve, making an exact comparison impossible (74, 91). With some mycobactins, growth follows a linear course after the initial lag, whereas with others the growth rate increases slowly during the earlier periods of measurement. Besides this, there is a very evident difference in the relationship between mycobactin concentration and growth response from one mycobactin to another (Fig. 1). Mycobactin S is outstanding in showing growth stimulation at concentrations as low as 0.3 ng/ml, whereas with others (e.g., mycobactin R) no stimulation of growth occurs below a concentration of 3 ng/ml. In all cases, near-maximal growth is attained at 30 ng/ml. These curves illustrate the impossibility of making a precise comparison of activity between every type of mycobactin. Snow and White (74) divided the mycobactins into two groups, each group showing sufficiently similar growth characteristics to allow a rough comparison to be made of their relative activities; however, mycobactins M and N and dihydro mycobactin P (all compounds having saturated side chains) should possibly be placed in a third, separate group. Differences in activity

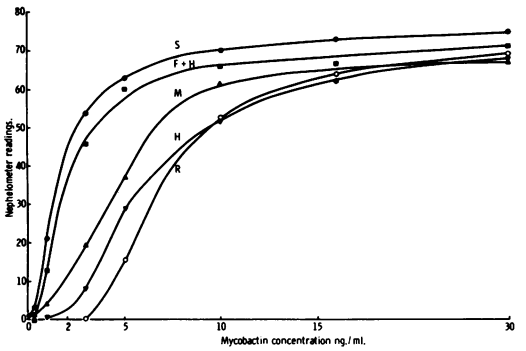



FIG. 1. *Growth of M. paratuberculosis in the presence of various concentrations of different mycobactins. Points show the increase in nephelometer reading after growth for 14 days; each point is the mean of three replicates. Symbols:* ●, *Mycobactin S;* ■, *mixture of mycobactins F and H from M. fortuitum;* ▲, *mycobactin M;* ▼, *mycobactin H;* ○, *mycobactin R.*

within each group are small, the maximal difference being about twofold. The most active compound at low concentrations is mycobactin S, but mycobactin F, which has only been obtained so far in admixture with a preponderance of mycobactin H, may possibly be even more active on its own than mycobactin S.

### Other Growth-Promoting Properties of Mycobactins

Apart from *M. paratuberculosis*, the only other mycobacterium known to be strictly dependent on mycobactin for growth is the "wood pigeon mycobacterium" (92). Since most other mycobacteria, except for *M. lepraemurium* and *M. leprae*, are readily cultured on laboratory media and are known to produce their own mycobactins, it is not surprising that they are usually unresponsive to exogenous mycobactins. However, stimulations of growth have been recorded with *M. phlei* (65), *M. smegmatis* (106), and *M. tuberculosis* growing under unfavorable conditions in a medium containing serum at a *p*H of 7.8 to 8.0 (47). Mycobactins can also replace sideramines as growth factors for various sideramine-dependent organisms such as *A. terregens*, *A. flavescens*, and *A. citreus* (15, 52, 65). The reverse, however, is not true. Mycobactin cannot be replaced as a growth stimulant for *M. paratuberculosis* by ferrichrome, Terregens factor, or coprogen (50); nor can the growth be stimulated by high concentrations of hemin.

Although *M. paratuberculosis* has this highly specific requirement of mycobactin for growth, Morrison has found conditions under which various strains of the organism will grow at least slowly without supplementation by mycobactin. These involve the use of unusually acid conditions, preferably around *p*H 5.0, and growth in a simple glucose-containing medium which has been autoclaved at *p*H 5.5 (34, 50). The autoclave treatment produces unidentified compounds having absorption maxima at 230 and 290 nm. Although strains of *M. paratuberculosis* may be cultivated for many years under these conditions, they still show an absolute dependence on mycobactin when they are returned to the more normal conditions of growth. Dependent strains of this organism, in contrast to other mycobacteria, seem to have a true genetic defect, being unable to produce mycobactin. When they were grown without added mycobactin, even in medium of low iron content which should stimulate mycobactin production, no trace of mycobactin-like material was found by biological assay or by tests for hydroxylamine (50). The extreme dependence on exogenous mycobactin and the high specificity of the mycobactins to the mycobacteria present problems in explaining the florid growth of *M. paratuberculosis* in intestinal tissue in Johne's disease. This question has been discussed at some length by Hanks (34). Very acidic conditions existing locally in the vacuoles of cells may permit growth without the intervention of mycobactin. On the other hand, it is not inconceivable that sufficient supplies of mycobactin may reach the organism through the blood stream. Amid the varied bacterial flora to be found in the alimentary tract or elsewhere in the ruminant there could be nonpathogenic mycobacteria capable of producing mycobactins. The aqueous solubility of the mycobactins is certainly low, but the amount required for growth of *M. paratuberculosis* is so small that this limitation presents no obstacle. If mycobactin is available at the necessary concentration of 1 to 10 μg/liter of body fluids, Hanks' work shows that penetration to the intracellular mycobacteria would be quite feasible.

### Mycobactin Inhibitors

Attempts have been made to find antagonists for mycobactin. An early report of competitive antagonism of the inhibitory effects of D-cycloserine on the growth of *M. phlei* (76) have not been substantiated (106). The only example of an antagonistic action so far known is that due to the chromic mycobactins (73). The growth of *M. paratuberculosis* on a medium supplemented with mycobactin P is inhibited by the presence of chromic mycobactin P (Table 3). The amount of the chromic complex required for inhibition increases with the concentration of mycobactin present.

TABLE 3. *Antagonism by chromic mycobactin P of the growth-promoting effect of mycobactin P towards M. paratuberculosis*[a]

| Chromic mycobactin P (μg/ml) | Mycobactin P (μg/ml)[a] | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.01 | 0.03 | 0.1 | 0.3 | 1.0 |
| 0 | 0 | 46 | 45 | 44 | 46 | 47 |
| 0.5 | | 34 | 38 | 39 | 39 | 37 |
| 1 | | 12 | 20 | 34 | 38 | 37 |
| 5 | | 1 | 0 | 2 | 7 | 33 |

[a] Values represent mean increases in nephelometric readings after growth for 12 days.

Mycobactins M and N show an unusual growth-depressing effect. With most mycobactins, growth of *M. paratuberculosis* increases with increasing mycobactin concentration over a limited range, but beyond a concentration of about 30 ng of mycobactin per ml growth remains constant and unaffected by further mycobactin, even up to quite high levels. With mycobactins M and N, however, growth is noticeably depressed at higher concentrations. Investigation of this effect led to the unexpected observation that when either of these mycobactins was present together with mycobactin P or other P-type mycobactins the growth of *M. paratuberculosis* with the mixture was less than with the same concentration of either separately (74). This curious effect is illustrated in Fig. 2. The depression in growth caused by high concentrations of mycobactin M was apparently due to a prolongation of the initial lag; at 12 days the amount of growth with 1,000 ng of mycobactin M per ml was considerably less than with 10 ng/ml, but beyond this time the growth curves were parallel. The growth curve in the presence of 10 ng of mycobactin P per ml was higher than with 1,000 ng of mycobactin M per ml but had a shallower slope; the growth curve for the mixture showed a prolonged lag, followed by a slope similar to that found with 10 ng of mycobactin P per ml. Thus, at any selected time of incubation there appears to be a mutual inhibition of growth, but strictly it should be described as an interaction leading in some way to a delay in the onset of growth. A similar but less striking effect was observed when the concentrations of mycobactins M and P were both 10 ng/ml. The growth curve for the mixture was lower than the curves for the individual mycobactins, presumably because of a delay in the onset of growth, but the slope of the curve was identical with the slope of the curve for mycobactin P alone.

In view of this result, mycobacteria not dependent on mycobactin were grown in medium containing mycobactin M or N (Fig. 3). The M-type mycobactins at a fairly high concentration inhibited the growth of *M. kansasii* and *M. tuberculosis*. As with *M. paratuberculosis*, the effect was at least partly a delay in the onset of growth. The growth rate in the presence of the mycobactins increased with time, but even after 11 days it was still below the control growth rate.

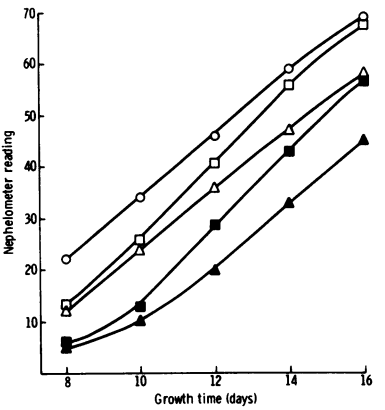



FIG. 2. *Mutual inhibitory effects of mycobactins M and P on the growth of M. paratuberculosis. Concentrations of mycobactins (ng per ml): ⊙, 10 P; ⊡, 10 M; △, 10 P + 10 M; ■, 1,000 M; ▲, 10 P + 1,000 M. Each point is the mean of three replicates.*

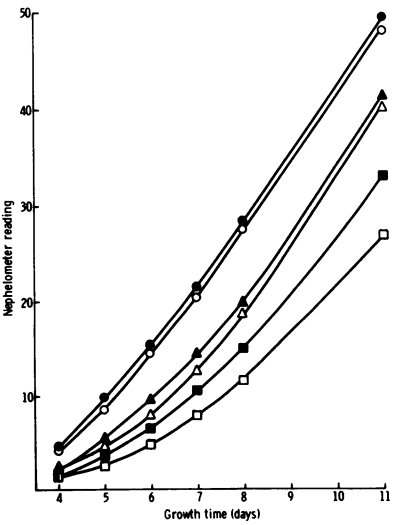



FIG. 3. *Effect of mycobactin M on the growth of M. tuberculosis. Symbols: ⊙, no addition; □, 3 μg of mycobactin M added per ml; △, 3 μg of mycobactin M and 3 μg of mycobactin P added per ml. Filled points show corresponding readings where the medium was supplemented with ferric iron (15 μg/ml). Each point is the mean of eight replicates.*

In this experiment, the mycobacteria were grown in a medium to which no iron was added; under these conditions, the endogenous production of mycobactins was enhanced. The effect was also seen when iron was added to the medium, growth being somewhat increased both with and without mycobactin M. The addition of mycobactin P reduced, but did not abolish, the action of mycobactin M.

It is interesting that this growth-inhibitory effect has been found only with mycobactins M and N, which differ considerably from other mycobactins in their chemical structure.

## CHEMICAL STRUCTURE

### Nomenclature

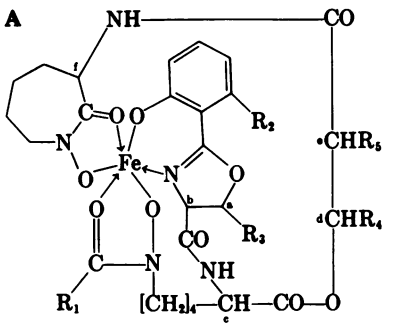
Systematic nomenclature of the mycobactins has not been attempted; chemical structural names would be impossibly clumsy and trivial names are unavoidable. Mycobactins occur as families in which the main nucleus is constant; the members of each family form a homologous series with side chains of various lengths. There is usually one preponderant side-chain length with smaller amounts of higher and lower homologues. The mycobactins have been distinguished primarily by letters. Each letter represents a family of mycobactins having the same nucleus. Individual members of the family can then be distinguished by a figure indicating the number of carbon atoms in the side chain (93). Methods are available for separating the mycobactins into separate families, but separation of individuals within the family is much more difficult and usually has not been attempted.

All the known mycobactins have the same basic molecular pattern with variations in substitution at five points in the molecule. However, there are two main types which are sufficiently different to make it convenient to consider them separately. They will be referred to as the P-type and M-type (named after representative mycobactins). The general structure of the mycobactins is shown in Fig. 4; the substituents present in the identified mycobactins are also shown.

### Chemical Degradation

The structure of mycobactin P was originally established by chemical degradation (69, 70, 71) and the methods have been subsequently extended

A




B

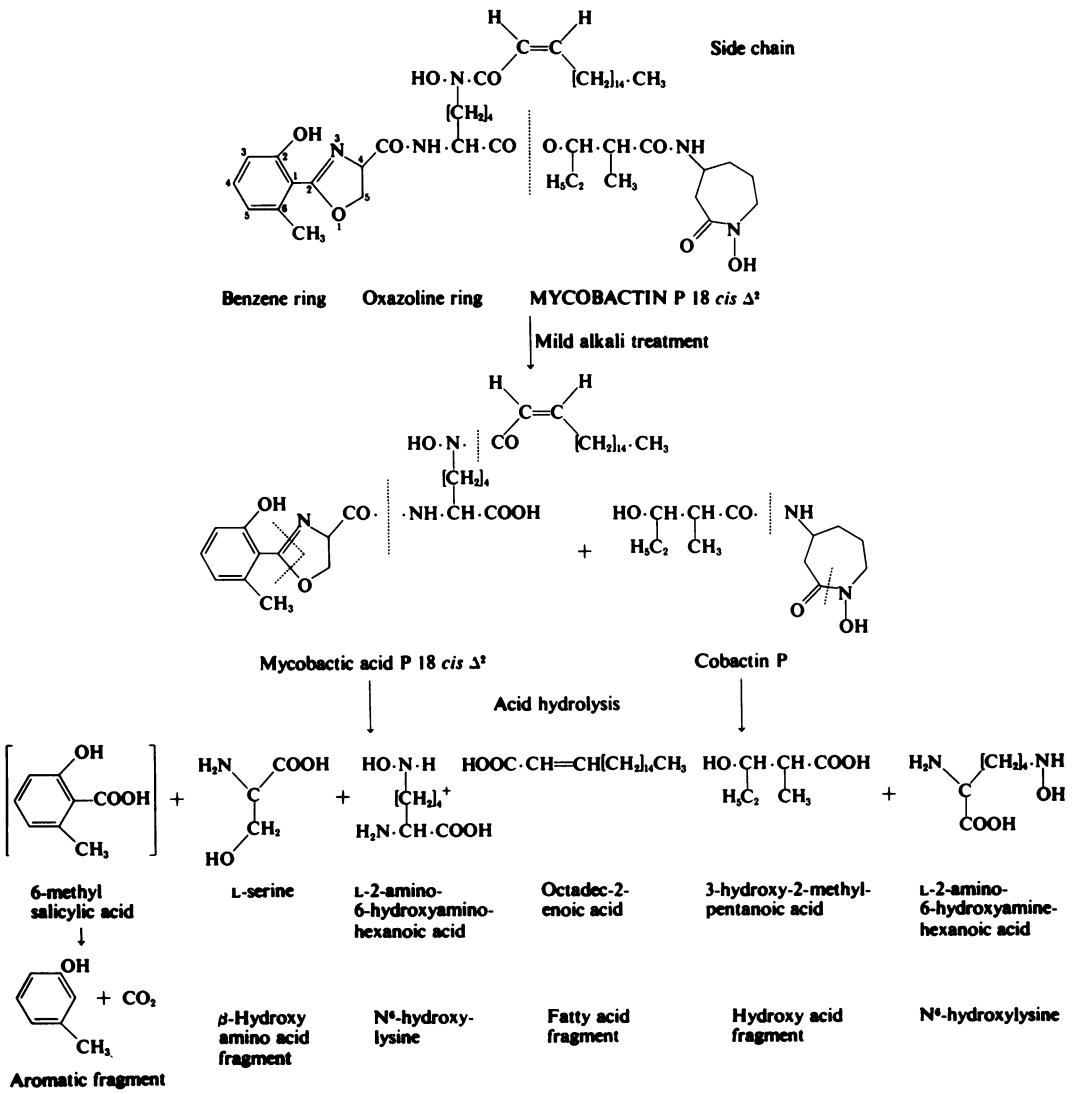
| Mycobactin | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | a | b | c | d | e | f |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $A^+$ | **13Δ** | $CH_3$ | H | $CH_3$ | H | | | | | | |
| F* | **17**, 15, 13, **11**, 9Δ | H | $CH_3$ | $CH_3$ | H | Threo | | | *S* | ( ) | L |
| H | **19**, **17**Δ | $CH_3$ | $CH_3$ | $CH_3$ | H | *R* | L | L | *S* | ( ) | L |
| M | 1 | H | $CH_3$ | 18, **17**, 16, 15‡ | $CH_3$ | | | | *R*∂ | *S*∂ | |
| $N^+$ | 2 | H | $CH_3$ | 18, **17**, 16, 15‡ | $CH_3$ | | | | | | |
| P | 19, **17**, 15 *cis*Δ[1]*n* | $CH_3$ | H | $C_2H_5$ | $CH_3$ | ( ) | L | L | *S* | *R* | L |
| R | **19**Δ | H | H | $C_2H_5$ | $CH_3$ | ( ) | L | L | *R* | *S* | L |
| S | 19, **17**, **15**, 13*cis*Δ | H | H | $CH_3$ | H | ( ) | L | L | *S* | ( ) | L |
| T | 20, **19**, 18, 17<br>20, **19**, 18, 17Δ | H | H | $CH_3$ | H | ( ) | | | *R* | ( ) | L |

FIG. 4. *(A) General structure of the ferric mycobactins. Side chains $R_1$ are alkyl groups having the number of C atoms shown; double bonds are indicated where they are known. (B) Figures show the main types of side chain; those of greatest abundance are shown in bold type. Asymmetric centers are labeled a–f. Blanks indicate that the configuration has not been determined. Key: ( ), lack of asymmetric center; *, structure inferred from mixture with mycobactin H; †, tentative structures; ‡, saturated alkyl groups having the number of C atoms shown; §, relative configuration at d and e is erythro—absolute configuration is uncertain.*

to other mycobactins; nuclear magnetic resonance and mass spectrometric measurements have also been used.

In discussing the structures of the different mycobactins an outline must be given of the main degradation products (Scheme 1). These are illustrated with reference to mycobactin P, but other mycobactins undergo similar reactions. Broken lines indicate the points of cleavage; the different parts of the original molecule and the degradation products are labeled for reference in the subsequent discussion. The first degradation splits the ester link to give two products known as the mycobactic acid and the cobactin. Each of these can be further split by acid hydrolysis.

With the P-type mycobactins, the mycobactic acid unit yields the following. (i) An aromatic acid, either salicylic acid or 6-methyl salicylic acid; the latter undergoes further degradation to *m*-cresol and carbon dioxide. (ii) A $\beta$-hydroxy amino acid, either serine or threonine. (iii) A mixture of homologous long chain fatty acids, usually with a double bond adjacent to the carboxyl group. Under conditions of acid hydrolysis isomerization of this double bond occurs; the fatty acid fragment may be isolated with retention of the original configuration by oxidation of the mycobactic acid with periodate (71). (iv) $N^6$-hydroxylysine. The cobactin unit yields: (v) a $\beta$-hydroxy acid, either 3-hydroxy butyric acid or 3-hydroxy-2-methylpentanoic acid; and (vi) a second molecule of $N^6$-hydroxylysine. The degra-




SCHEME 1. *Main degradation products of mycobactin P.*

dation of M-type mycobactins is similar except iii and v. With these mycobactins the mycobactic acid gives no long-chain fatty acids on hydrolysis. Short-chain volatile acids are probably liberated but these have not been identified with certainty.

The hydrolysis of the cobactins M and N gives a homologous series of long-chain β-hydroxy acids (74).

### Chemical Differences Between Mycobactins

Consideration of the degradation products and of various physical properties allows certain generalizations to be made about the mycobactins so far known.

In the P-type mycobactins (Fig. 4), a long alkyl chain is always present at $R_1$ and in most cases the side chain has a double bond adjacent to the carbonyl group. The only known exception is in mycobactin T in which some members of the family probably have saturated alkyl side chains (72). The lengths of the side chains, their relative abundance, and the presence of the double bond have been established for the different mycobactins, but only with mycobactin P has the nature of the side chain been fully proved. Here, the main component has been purified and shown to have the n-*cis*-octadec-2-enoyl side chain. This individual compound is named mycobactin P 18-*cis*-$\Delta_2$ (93). In other mycobactins, the possibility of branching of the side chains has not been excluded.

The differences between the nuclei of known P-type mycobactins result from varying combinations of the following possibilities: (i) the presence or absence of a methyl group at position 6 in the benzene ring; (ii) the presence or absence of a methyl group at position 5 in the oxazoline ring; (iii) the presence of either 3-hydroxybutyric acid or 3-hydroxy-2-methylpentanoic acid as the β-hydroxy acid fragment; and (iv) variations in the configuration of asymmetric centers in the β-hydroxy acid fragment. The last is the most elusive difference. It is best illustrated by mycobactins S and T in which the sole difference between the nuclei probably lies in the optical configuration of the 3-hydroxy-butyric acid (94). The 3-hydroxy-2-methylpentanoic acid units of mycobactins P and R are enantiomers, both having the *erythro* structure (74).

In the M-type mycobactins, a long alkyl chain occurs in the hydroxy acid fragment together with an α-methyl group. As with those P-type mycobactins that have two asymmetric centers in this fragment, the configuration is *erythro*. In contrast to the P-type mycobactins, the M-type have only a small alkyl group at $R_1$ (Fig. 4). Only two members are known so far, mycobactin M where $R_1 = CH_3$ and mycobactin N where $R_1 = C_2H_5$; both have a methyl group at position 5 in the oxazoline ring and lack a methyl group in the benzene ring (74).

## PHYSICAL AND CHEMICAL PROPERTIES OF THE MYCOBACTINS AND THEIR DERIVATIVES

### General

Mycobactins are all white powders showing microcrystalline structure by X-ray powder photography, though none has been crystallized in the conventional sense. Attempts at crystallization lead to the separation of the mycobactin in a gelatinous form. The solids have definite melting points and are stable to heat and to air up to at least 100 C; they show a characteristic apple-green fluorescence in ultraviolet light. Their solubility in water is low, being in the range 5 to 15 μg/ml at 20 C. They are extremely soluble in chloroform, fairly soluble in ethanol (about 2% at 20 C), and less soluble in other alcoholic solvents such as methanol or propylene glycol. They have little solubility in less polar solvents such as ether, benzene, or aliphatic hydrocarbons. They are moderately stable to acids but break down easily under alkaline conditions.

The characteristic ultraviolet absorption spectra of the mycobactins arise from two parts of the molecule. The main spectral features are due to the 2-(*o*-hydroxyphenyl)-oxazoline structure, but there is a considerable absorption contribution below 300 nm from the $\Delta^2$-acylhydroxamic acid group —CH=CH—CO·N(OH)— present in many mycobactins (70). The mycobactins show two main types of absorption spectrum according to the substitution in the benzene ring: (i) no methyl group present, $\lambda_{max}$ (in methanol) 243, 249, and 304 nm with inflection at 258 nm; and (ii) methyl group at position 6, $\lambda_{max}$ 250 and 311 nm with shoulder at 254 nm and inflection at 265 nm. The presence or absence of a methyl group in the oxazoline ring hardly affects the absorption spectrum.

Where the acyl group of the hydroxamic acid center in the mycobactic acid is saturated, as in mycobactins M and N and in dihydromycobactin P, the absorbances of the maxima at the lower wavelengths are lower and the peaks are more clearly defined (Fig. 5).

Other properties have been studied in detail only with mycobactin P (27, 70) but are probably shared by all the mycobactins. Careful titration of mycobactin P with alkali in methanol shows the presence of two acidic groups, identified as the two hydroxamic acids. Their *p*K cannot be readily determined in mycobactin P itself because of its low aqueous solubility, but in cobactin P

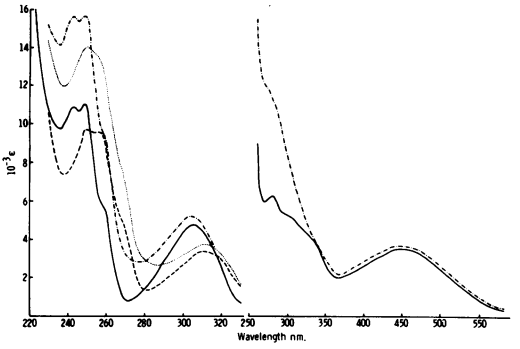



FIG. 5. *Absorption spectra of mycobactins (curves on left) and ferric mycobactins (curves on right). Symbols: ——, Mycobactin M; -·-·, mycobactin S; -----, dihydromycobactin P; ····, mycobactin P (all as solutions in methanol).*

the hydroxamic acid has *p*K 9.1. The two hydroxamic acid groups can be acetylated giving diacetylmycobactin P, which has no groups titratable under comparable conditions; the phenolic group is much less acidic. The diacetyl compound is unchanged in absorption spectrum but reacts differently with ferric chloride, giving a violet color due to the phenolic group rather than the reddish color of the ferric hydroxamic acids.

Mycobactin P also shows a single weakly basic group when titrated with perchloric acid in anhydrous acetic acid, due to the nitrogen atom of the oxazoline ring. In line with this property, mycobactin P forms salts with acids under anhydrous conditions, the hydrochloride and picrate having been isolated in a solid form. As would be expected, protonation of the oxazoline nitrogen in mycobactin P hydrochloride causes a marked shift in the ultraviolet absorption maxima. The The hydrochloride dissolved in chloroform can be reconverted to the parent compound by repeatedly washing the solution with water.

Catalytic hydrogenation of mycobactin P causes saturation of the double bond in the side chain. The resultant dihydromycobactin P resembles the parent compound closely, but the absorption spectrum is modified as discussed above (74).

### Nuclear Magnetic Resonance (NMR) Spectra

NMR measurements have proved of particular value in distinguishing and identifying mycobactins (32). In spite of the complexity of the structures and the presence of some 70 protons, many salient points of the molecule can be clearly distinguished. The numerous $CH_2$ groups which form part of saturated aliphatic chains are all grouped together in a series of large peaks, but the substituents which vary from one mycobactin to another give distinctive peaks. Thus the NMR spectrum shows the presence or absence of a methyl group in the benzene ring or in the oxazoline ring. It also shows the nature of the groups in the hydroxy acid fragment. In mycobactins M and N, resonances are found corresponding, respectively, to the protons of the acetyl or propionyl groups at the hydroxamic acid center in the mycobactic acid moiety. Inspection of the spectra for the presence or absence of five selected peaks allows a distinction to be made between all the mycobactins so far isolated except for mycobactins S and T, in which the nuclei have identical substituents and differ solely in the configuration of asymmetric centers.

### Metal Complexes

**Ferric complexes.** The most significant chemical properties of the mycobactins are their ability to chelate with metals and their strong selectivity for ferric iron. As with the water-soluble sideramines, the stabilities of the complexes with ferric iron are far higher than with any other metal ion at physiological *p*H values. Stability constants of several ferrioxamines and compounds related to ferrichrome have been measured and have extraordinarily high values of approximately $10^{30}$ (1). The methods used for measurements on these compounds are not easily adaptable to the water-insoluble mycobactins. However, measurements have been made of the relative affinities of desferrioxamine B and mycobactin P for ferric iron in ethanolic solution; indications of their relative affinities in aqueous solution have also been obtained by experiments using the two-phase system of chloroform and *p*H 7.0 buffer. Both methods showed that ferric iron had an even greater affinity for mycobactin P than for desferrioxamine B (74). With the ferrioxamines and compounds of the ferrichrome type, the nature of the chelation complexes is well understood. These compounds have three hydroxamic groups so situated in the molecule that they can assume an arrangement in which their six oxygen atoms form the apices of an octahedron with the iron atom at the center. In this condition, the molecule has no net charge, and all six coordination valencies of the metal ion are satisfied. Reaction of the desferricompound with a ferric ion involves the displacement of three protons from the hydroxamic acid groups. A full, three-dimensional structure has been worked out for ferrichrome A by X-ray crystallography (104). The mycobactins

have only two hydroxamic acid groups, but have a third iron-binding center comprising the phenolic hydroxyl group and the nitrogen atom of the oxazoline ring. All three centers are apparently engaged in chelation with the iron atom. Models show that they can be accommodated around the iron atom in a similar way to the trihydroxamic acid chelates except that the iron atom has five oxygen atoms and one nitrogen atom in its co-ordinating positions. Since all three chelating centers in the mycobactins are different and unsymmetrical, there are 16 different ways in which they could be arranged around the central atom. However, the fact that these groups are connected by a bifurcated chain imposes a constraint on the number of structures that are stereochemically possible. Three structures can be made with space-filling molecular models in which there is no undue strain and the substituent groups seem conveniently disposed (71). A few other structures can be made as models but they have various unsatisfactory features. The actual disposition of the chelating groups should be settled by X-ray crystallography of ferric mycobactin P, which is being undertaken currently.

Ferric mycobactin P readily crystallizes as thin reddish-brown hexagonal prisms (71), but none of the other ferric mycobactins has shown any sign of crystallization. All the ferric mycobactins show an absorption peak at 450 nm, the height of which is an important criterion of purity during purification procedures. The absorption spectra are somewhat lacking in distinctive features at lower wavelengths, but show differences between mycobactins with or without a methyl group in the benzene ring (Fig. 5).

The readiness with which ferric iron will chelate with the mycobactins can be utilized as a means of estimating low concentrations of ferric iron in aqueous solution. A neutral aqueous solution is shaken with a small volume of a chloroform solution of mycobactin. The layers are separated and the $E_{450}$ of the dried chloroform layer is determined, if necessary after concentration. This method will readily determine ferric iron at a concentration of 10 $\mu$g/liter or less. The same procedure can also be used to strip ferric iron from aqueous solutions, giving extremely low concentration levels.

**Aluminum and gallium complexes.** The mycobactins form aluminum complexes by reaction with aluminum chloride in alcoholic solution; complexes are not formed in the presence of water, but the complexes once formed are stable to water. As with the ferric complexes, aluminum

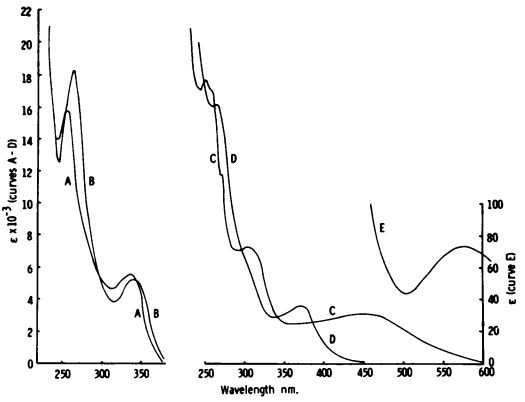



FIG. 6. *Absorption spectra of metal complexes of mycobactins. Curve A, aluminum mycobactin S; curve B, aluminum mycobactin P; curve C, vanadyl mycobactin P; curves D and E, chromic mycobactin P. (Aluminum complexes as solutions in methanol; other complexes in ethanol.)*

mycobactin P readily gives crystals [colorless, thin hexagonal prisms (27)], but other aluminum mycobactins have not been crystallized. The aluminum complexes have well marked absorption maxima (Fig. 6). Aluminum mycobactins having a methyl group in the benzene ring give maxima in methanol at 265 and 342 nm, whereas those without this methyl group have maxima at 257 and 336 nm. Solutions of the aluminum complexes show a strong violet fluorescence in ultraviolet light. The conversion of a mycobactin to its aluminum or other metal complex involves the change from a very open, unconstrained structure to a highly ordered and restricted structure containing eight interlocking rings. This is reflected in the change in optical rotation in chloroform from $[\alpha]_D^{25} - 19°$ for mycobactin P to $[\alpha]_D^{15} + 376°$ for its aluminum complex (27). The aluminum complexes of the mycobactins have proved useful for characterization and identification since they are sufficiently volatile to give satisfactory mass spectra. In these spectra, the parent ions can be readily detected and provide an exact measure of the molecular weights. This is particularly valuable since most mycobactins are mixtures of several homologues. Since these components differ only in their side chains, they will have very similar volatilities and the ratios of the peak heights in the mass spectrum give a reasonable measure of the relative abundance of the components (Table 4). In some cases, this has been confirmed by gas chromatographic measurements on derivatives of the fatty acids liberated by degradation of the mycobactin (74).

TABLE 4. *Mass spectrometry of the aluminum complexes of various mycobactins*

| Aluminum mycobactin | Determinations[a] |
|---|---|
| A | *m/e* 809<br>RA 100[b] |
| H | *m/e* 907 879<br>RA 50 50 |
| M | *m/e* 909 895 881 867 853<br>RA Tr 2 55 25 18 |
| P | *m/e* 921 907 893 879 865 837<br>RA Tr 1 90 1 7 Tr |
| R | *m/e* 907 879<br>RA 100[b] Tr |
| S | *m/e* 879 851 823 795<br>RA 9 36 46 9 |
| T | *m/e* 895 893 881 879 867 865 853 851<br>RA 12 10 24 25 7 8 7 7 |

[a] Abbreviations: RA, approximate relative abundance of molecular species; Tr, trace. *m/e* Values are for parent ions.

[b] Approximate.

Another useful distinguishing feature of the mass spectra of the aluminum mycobactins is the peak for the first prominent fragment ion. With a P-type mycobactin, this peak occurs at a mass number corresponding to a loss of the side chain beyond the $\alpha\beta$-double bond (93). Thus, although there are several parent peaks (corresponding to homologues having different lengths of side chain), there is only one first daughter ion which defines the molecular weight of the nucleus (*see* Table 5). This helps to establish the type of substituents present in the nucleus. The M-type mycobactins are readily distinguished by mass spectrometry since their aluminum complexes undergo an entirely different breakdown. These mycobactins also give series of parent peaks corresponding to homologues having different lengths of side chain. However, because of the different nature of the side chain attachment in these mycobactins the degradation in the electron beam takes a different course. The whole cobactin fragment carrying the variable side chain is lost, leaving a single ion characteristic of the mycobactic acid part of the nucleus (74).

Gallium mycobactin P has been crystallized (73) and shows very similar properties to the aluminum complex. Treatment of mycobactin P with indium chloride under the conditions used for preparing the aluminum and gallium complexes gives no reaction.

**Chromic complexes.** Mycobactin P reacts less readily with chromic chloride than with aluminum or gallium chloride. Treatment for 1 hr at 60 C gives the chromic complex, but with some degradation. The complex, purified by chromatography, can be crystallized (73). Its absorption spectrum is shown in Fig. 6. A noncrystalline complex was prepared similarly from mycobactin S.

**Dissociation of tervalent metal complexes under acid conditions.** The formation of the tervalent metal complexes of the mycobactins involves the loss of three protons, two from the hydroxamic acid groups and one from the phenolic hydroxyl group. The reaction may be represented by equation A, where $MyH_3$ represents mycobactin with its three replaceable hydrogen atoms and M is the tervalent metal

$$MyH_3 + M_3^+ \rightleftharpoons MyM + 3H^+ \qquad (A)$$

Many tervalent metal ions have been investigated but only the complexes already described are readily formed. From equation A, it is evident that dissociation of the complexes should occur under acid conditions. This has been demonstrated in all cases, and provides a means of regenerating the mycobactins from their metal complexes. The normal method is to shake a chloroform solution of the metal complex with 5 M hydrochloric acid. The metal chloride forms in the aqueous layer and metal-free mycobactin is left in the chloroform layer. The mycobactins are stable to acid under these conditions. With the ferric complexes, a relationship was found between acid concentration and the degree of dissociation. Under controlled conditions, the extent of dissociation varies from one type of ferric mycobactin to another and may possibly be used as a means of distinguishing mycobactins (74). The chromic complex is much more resistant than other complexes to dissociation by acid, and concentrated hydrochloric acid is required to release the chromic ion (73).

**Vanadyl complexes.** Apart from the complexes with tervalent ions, the mycobactins also form brown-purple complexes with vanadate (73). These apparently are vanadyl complexes, their formation being represented by equation B

$$MyH_3 + VO_3^- + H^+ \rightleftharpoons MyVO + 2H_2O \qquad (B)$$

This equation shows that the formation of the vanadyl complex differs from the reaction with tervalent ions. Increasing hydrogen ion concentration should tend to increase the stability of

the complex rather than bring about dissociation. This agrees with experimental results. Chloroform solutions of vanadyl mycobactins are not dissociated by shaking with the strongest acids. Acid treatment changes the color of the complexes to a deep blue, which reverts to the normal plum color when the chloroform layer is washed with water. The absorption spectrum of vanadyl mycobactin P is shown in Fig. 6. The complex is difficult to isolate in a solid form. Vanadyl complexes are also formed by the water-soluble sideramines of the ferrioxamine and ferrichrome groups. These are in many ways parallel to the vanadyl mycobactin complexes and have been studied in greater detail (73).

The mycobactins and their derivatives are conveniently detected on thin-layer chromatogram plates by spraying with an acid solution of ammonium vanadate. The intense blue spots are more readily seen than the red-brown color of the ferric complexes (94).

**Copper mycobactin P.** The copper complex is formed as an amorphous green precipitate by addition of ethanolic cupric chloride to an ethanolic solution of mycobactin P (27). It contains 1 atom of copper per molecule of mycobactin P. The copper complex has considerably lower stability than the ferric complex since the metal can be displaced by treatment with hydrogen sulfide; the ferric complex is unchanged by the same treatment. The copper complex played an important part in the first purification of mycobactin P.

## BIOSYNTHESIS

The mycobactins present an interesting problem in biosynthesis since the mycobactin molecule is apparently derived from six component parts coming from various biochemical pathways. An understanding of the biosynthesis will require a study of the derivation of each of these parts, followed by experiments to show the order in which they are assembled and the control processes which keep the various biosyntheses in step. So far very little has been done.

### $N^6$-Hydroxylysine

The most biochemically unusual product of acid hydrolysis is $N^6$-hydroxylysine. This is represented twice in the molecule, once in an acyclic form and once as a cyclic structure. In both cases, the hydroxyamino group is acylated to give a secondary hydroxamic acid. The biosynthesis of a similar component, $N^5$-hydroxyornithine present in ferrichrome, was studied by Emery (26). Using $^{14}C$-labeled compounds, Emery demonstrated the incorporation of ornithine, $N^5$-hydroxyornithine, and $N^5$-acetyl-$N^5$-hydroxyornithine into ferrichrome by *Ustilago sphaerogena*. The second of these compounds was incorporated to the greatest extent over a 6-hr incubation period, but the last was the best precursor in a 24-hr experiment. $N^5$-Acetylornithine was not appreciably incorporated. There was some difficulty in the experiment because of the instability of $N^5$-hydroxyornithine in the medium. This also precluded finding the hydroxyamino compound in the cells during the biosynthesis of ferrichrome. Emery concluded that the biosynthetic pathway to ferrichrome involved $\delta$-N-hydroxylation of ornithine, followed by acetylation and subsequent incorporation of hydroxamate residues into the hexapeptide ring. Tateson (78) studied the biosynthesis of the corresponding $N^6$-hydroxylysine component of mycobactin. With *M. phlei* and *M. smegmatis*, $^{14}C$-labeled lysine was incorporated in good yield into both $N^6$-hydroxylysine residues in mycobactin; this was also shown earlier for *M. phlei* by Birch and his colleagues at Manchester (1). However, $^{14}C$-$N^6$-hydroxylysine was not taken up at all by *M. phlei* cells, in contrast to the uptake and utilization of $N^5$-hydroxyornithine by *U. sphaerogena*.

In the experiments with labeled lysine, Tateson was unable to detect the presence of $N^6$-hydroxylysine within the cells during the biosynthesis of mycobactin, but this negative finding does not exclude its function as an intermediate; its steady state concentration might lie below the limits of detection. During the experiments with *M. phlei* and *M. smegmatis* using $^{14}C$-lysine, a labeled water-soluble product was detected in the medium. The products from the two organisms had similar chromatographic properties but their identity was not otherwise established. The labeled product from *M. phlei* was readily incorporated into mycobactin P by the same organism, and since this incorporation was not diminished in the presence of unlabeled lysine which is known to enter the cell readily, the product was probably not entering mycobactin P through prior breakdown to lysine. Tateson did not fully identify the metabolite but showed it to be an $N^6$-derivative of lysine that gave lysine on acid hydrolysis. The possibility that the compound was an intermediate in the biosynthesis of mycobactin P was strengthened by the observation that its production was repressed by an increase in iron concentration that also represses mycobactin production. This opens the possibility that the hydroxamic acid groups in mycobactin do not arise from direct hydroxylation of the $\epsilon$-amino group of lysine. The first step may be

$N^6$-acylation followed by hydroxylation of the amide at a later stage, but more experiments are needed to test this point.

### Hydroxy Acid Unit

Apart from mycobactins M and N, the mycobactins all contain a 3-hydroxybutyric acid residue or a 3-hydroxy-2-methylpentanoic acid residue. These would appear to be derived from two acetate or two propionate residues, respectively (4). Tateson (78) showed that labeled propionate was incorporated by *M. phlei* into mycobactin P very largely in the hydroxy acid unit. With *M. smegmatis*, acetate was strongly incorporated into the 3-hydroxybutyric acid unit of mycobactin S with no appreciable incorporation into the amino acid units. Predictably, there was strong uptake of the label into the ether-soluble hydrolysis products of mycobactin S, presumably into the long-chain fatty acid unit.

### Aromatic Acid Unit

All the known mycobactins contain either a salicylic acid or a 6-methylsalicylic acid residue. It seems likely that these acids are direct precursors since salicylic acid is found in *M. tuberculosis* and *M. smegmatis* (63) which give mycobactins with a salicylic acid residue, and 6-methylsalicylic acid is found in *M. phlei* which gives mycobactin P, having the methyl-substituted aromatic acid (64). I am indebted to C. Ratledge for details of unpublished experiments demonstrating the incorporation of salicylic acid into mycobactin S. $^{14}$C-salicylic acid was added to a growing culture of *M. smegmatis*. After 6 days, 8% of the $^{14}$C had been taken up by the cells; 90% was extracted in the mycobactin fraction, all the label being in the salicylic acid moiety. As the amount of $^{14}$C-salicylic acid was small, its uptake must have been against the efflux of salicyclic acid that is normally observed when *M. smegmatis* is grown on a medium deficient in iron. The origin of the aromatic acids in the mycobacteria is still under investigation. In penicillia, 6-methylsalicylic acid is synthesized by the malonate-acetate pathway (14). However, as discussed elsewhere (72), salicylic acid would be expected to come from shikimic acid. Ratledge and Winder (64) showed that washed iron-deficient cells of *M. smegmatis* incubated with shikimic acid gave only salicylic acid and 3,4-dihydroxybenzoic after an 8-hr incubation period. Gibson and his colleagues demonstrated the production of 2,3-dihydroxy benzoic acid from isochorismic acid in *Aerobacter aerogenes* and *Escherichia coli* (97, 99, 100) and suggested isochorismic acid as a likely precursor of salicylic acid in *M. smegmatis*. Ratledge (62) showed that *M. smegmatis* incorporated $^{14}$C-shikimate into salicylic acid in good yield. $^{14}$C-acetate was incorporated to much less extent, and the addition of unlabeled shikimate further lowered the labeling of the salicylic acid. If salicylic acid and 6-methyl salicylic acid prove to have different biosynthetic origins in mycobacteria, the mycobactins will provide an example of biosyntheses directed to form similar end products from two quite different routes. Mostly, this biosynthetic difference exists between different species of mycobacteria, but in a strain of *M. fortuitum* two mycobactins are produced by the same organism—mycobactin H containing a 6-methylsalicylic acid residue and mycobactin F containing a salicylic acid residue (74). Here, the two biosynthetic routes apparently co-exist in the same organism.

The effect of iron and zinc in the medium on the production of salicylic acid by *M. smegmatis* has been studied in some detail (60, 63, 64). Ratledge concludes that salicylic acid is produced in response to iron deficiency and acts as an iron chelator. The relationship between stimulation of mycobactin production and stimulation of salicylic acid production by lack of iron is still unclear.

### Other Units in the Mycobactin Molecule

Acid hydrolysis of the mycobactins yields a 3-hydroxy-2-amino acid, either serine or threonine. This residue exists as an oxazoline ring in the mycobactin molecule. The oxazoline is presumably derived from the appropriate amino acid biosynthetically, but the mechanism of ring closure is unknown; the oxazoline structure is unusual in natural products. The other element common to the P-type mycobactins is the fatty acid side chain. There are usually several different lengths of side chain in any mycobactin. The commonest side chains have 18, 20, or 16 carbon atoms (including the carbonyl group) and are thus similar in length to the common fatty acids; they probably arise by a similar biosynthetic route. Most of the fatty acid residues in the mycobactins probably have the rather unusual *cis*-$\Delta^2$ unsaturation; the mechanism of introduction of this double bond is unknown.

## Significance of Structural Differences Among Mycobactins Produced by Different Mycobacterial Species

The varied mycobactins produced by different mycobacterial species differ relatively slightly in their growth-promoting action towards *M. paratuberculosis*. Why this structural variation

should occur and should be linked with species in the mycobacteria is worth considering (94). It may be that each mycobactin is most exactly suited in its physical and chemical properties to the needs of the species producing it, and has been fixed by mutation and natural selection. On the other hand, growth-factor requirements may be satisfied by a fairly general molecular pattern, and the various structural variants carry no special biological advantage. If so, the particular compound produced could depend upon the availability of biochemical intermediates, and mutation would favor the use of the most accessible substances from the metabolic pool. The second possibility would be the more compatible with the existence of aromatic units in the mycobactins derived from different metabolic pathways. The idea is in some ways parallel to Bu'Lock's concept of secondary metabolites (13). These are thought to arise as by-products of the normal biochemical processes of the cell rather than in response to the need for a particular chemical structure. In the case of the mycobactins, however, a biological requirement is postulated, but this may perhaps be met by any near approximation to the basic structure.

## MYCOBACTINS IN THE TAXONOMY OF MYCOBACTERIA

### Occurrence

The recognition that *M. phlei* and *M. tuberculosis* produced mycobactins differing in chemical structure led to the investigation of a number of other species of mycobacteria. All strains that could be grown on synthetic media and were indisputably mycobacteria were found to produce iron-chelating compounds of the mycobactin type. [This generalization does not include *M. paratuberculosis* grown under special conditions in which a mycobactin supplement is not required. Morrison (50) was unable to detect mycobactin in *M. paratuberculosis* cells by assay or by a hydroxamic acid test. In these laboratories, we have also failed to demonstrate mycobactin in *M. paratuberculosis* growing independently.] An organism designated *M. rhodocrous* (NCIB 8574) could only be grown on nutrient agar and gave no mycobactin; however, the assignment of this organism to the genus *Mycobacterium* is doubtful (31). No intensive search has been made for evidence of mycobactin production by other genera of bacteria, but so far no organism outside the genus *Mycobacterium* has been shown to produce mycobactins. At the present time, the mycobactins may be regarded as entirely characteristic of the mycobacteria.

In examining different strains of mycobacteria, the problem arose of distinguishing between the mycobactins that were produced, without necessarily isolating the fully purified growth factors. Thin-layer chromatography of the ferric complexes proved the most useful tool for this purpose. It soon showed many more mycobactins than the two at first isolated (93). More surprisingly, most strains produced mycobactins giving only a single major spot on the thin-layer chromatography plate with only quite slight traces of other red-brown spots. This led to a wider investigation of mycobacterial species and of the mycobactins they produced.

### Classification of Mycobacterial Strains Correlated with the Type of Mycobactin Produced

Classification of the mycobacteria has presented many problems. The most satisfactory systems have been based on Adansonian methods and there is now some degree of agreement on the main classes of mycobacteria. Tsukamura's classification (79), which has features common to classifications by Collins (23) and Wayne (90), was used as a basis in the search for different mycobactins. Tsukamura considered that 36 named strains could be divided into 10 clearly distinguished clusters by assessment of 95 varied characteristics. In later publications (80, 81), two other clusters have been added. Strains were selected from 9 of the 10 original clusters and every one produced a characteristic mycobactin, judged by evidence of thin-layer chromatography and in many cases by isolation of a pure compound. Seven of the nine produced one main type of mycobactin with only traces of others. One strain, *M. marinum*, produced two related mycobactins both in substantial amounts; these were seen as two quite separate spots on a thin-layer plate. The remaining strain, *M. fortuitum*, produced mycobactins giving a single spot. The product was shown by physical methods to contain a mixture of two types of mycobactin, but no means could be found to separate them. The structures of the mycobactins from these various strains were discussed earlier. The marked correlation between the taxonomic classification and the type of mycobactin produced by the organism was further strengthened by more detailed investigation of one of Tsukamura's clusters. Four named strains from the *M. marinum* group were all shown to give mycobactin M. Systematic examination of other clusters has not been attempted, but the possibility of the predictive value of the identification of a mycobactin was illustrated in one particular case. An unidentified mycobacterial strain was shown to produce mycobactin S, a mycobactin known to be characteristic

of *M. smegmatis*; the strain was in fact later identified by J. Marks as *M. smegmatis*. The use of mycobactins to provide a broad classification of mycobacteria remains an interesting possibility which can only be substantiated by much more extensive tests. As a taxonomic method, it may be contrasted with other methods that are based on detection of substances produced by mycobacteria. Among substances suggested for use in characterization are lipids (38, 48, 66, 77), esterases (21), and proteins detected by antigenic analysis (8, 75). All these methods involve separation procedures followed by the recognition of patterns among the separated components that are characteristic of different strains of mycobacteria. In developing these methods, the mycobacterial strains are first classified by conventional methods and then submitted to the test. The separation and detection methods are empirical since the chemical nature of the components is undetermined. Conditions can be chosen to increase or decrease the selectivity of the test as required, and some methods will show up small differences between closely related strains. A classification based on mycobactins would be more objective since each mycobactin has a chemically defined nucleus and most mycobacteria appear to produce one mycobactin preferentially.

## Practical Methods of Identifying Mycobactins for Taxonomic Purposes

The possibility of using mycobactins as a practical taxonomic tool depends on the feasibility of identifying them without enormous labor. The practical requirements have been discussed (74). For this purpose, mycobacteria must be grown in quantity on a medium of low iron content. A simple solvent extraction and solvent purification leads to a crude product containing 2 to 40% ferric mycobactin which is suitable for examination by thin-layer chromatography. This provides the main method of distinguishing the mycobactins and serves to separate all the known types except for mycobactins H and F. If further proof of structure becomes necessary, the ferric mycobactins must be purified. Very small amounts of the purified ferric mycobactin can be converted into the metal-free form and thence to the aluminum complex. The ultraviolet absorption spectrum of the metal-free material and the mass spectrum of the aluminum complex (*see* PHYSICAL AND CHEMICAL PROPERTIES OF MYCOBACTINS AND THEIR DERIVATIVES) then provide information on the substitution in the benzene ring and the molecular weight of the chemical nucleus stripped of its variable side chain. Mass spectrometry also indicates the chain lengths and relative proportions of the side chains, which can vary in the same type of mycobactin isolated from different but related strains (74). The information available by these various means is summarized in Table 5. Finally, the chemical structure of mycobactins can be checked, and the structure of any new mycobactins can probably be indicated by the use of nuclear magnetic resonance spectroscopy (32). The operations involved in isolating and identifying mycobactins are likely to be unfamiliar and perhaps daunting to bacteriologists,

TABLE 5. *Properties of mycobactins and their derivatives suitable for their characterization*

| Mycobactin | Source | Thin-layer chromatography[a] | | Substitution in benzene ring[b] | Aluminum complex (first prominent fragment ion in mass spectrum) |
|---|---|---|---|---|---|
| | | Method a | Method b | | |
| | | $R_R$ | $R_F$ | | *m/e* |
| R | *Mycobacterium terrae* | 1.00 | 0.71 | H | 668 |
| N | *M. marinum* | 0.93 | 0.63 | H | 446 |
| P | *M. phlei* | 0.82 | 0.64 | Me | 682 |
| K | *M. kansasii* | 0.65 | 0.56 | | |
| M | *M. marinum* | 0.60 | 0.49 | H | 432 |
| H | *M. thermoresistible* | 0.35 | 0.50 | Me | 668 |
| F | *M. fortuitum* | 0.33 | 0.50 | H | 654 |
| T | *M. tuberculosis* | 0.31 | 0.57, 0.63[c] | H | 640 |
| A | *M. aurum* | 0.22 | 0.39 | Me | 654 |
| S | *M. smegmatis* | 0.19 | 0.48 | H | 640 |

[a] Method a, Cyclohexane, *tert*-butanol (9:1 by vol) on $Al_2O_3$; method b, isopropanol on silica gel. $R_R = R_F$ expressed as a fraction of the $R_F$ of ferric mycobactin R.

[b] Substitution in the benzene ring was determined by the wavelengths of absorption maxima of the metal-free mycobactin or the aluminum complex.

[c] Double spot.

but they may well be less laborious than the battery of tests at present used in classification studies.

## BIOCHEMICAL FUNCTION OF THE MYCOBACTINS

### Relationship of Mycobactins to Water-Soluble Sideramines

The growth-promoting effects of the mycobactins were discussed previously. No direct work has been done on their biochemical function, but some speculations can be made in the light of work on other iron-chelating compounds. The most direct analogy is with the sideramines (9). The term sideramine has not been precisely defined but the originators use the term to embrace compounds having the following characteristics. (i) They are natural products containing a single iron atom in each molecule strongly chelated to three hydroxamic acid groups. (ii) They promote the growth of dependent organisms such as *A. terregens* or *Pilobolus kleinii*. (iii) They are able to reverse competitively the inhibitory action of iron-chelating antibiotics (collectively known as the sideromycins) towards nondependent bacteria such as *Bacillus subtilis*. Within this category, the following compounds have been included: ferrichrome and various chemically related compounds (103) the ferrioxamines, coprogen, and Terregens Factor. These compounds comprise at least two chemically different types whose structures are exemplified by ferrichrome (67) and ferrioxamine B (10; Fig. 7). The structures of coprogen and Terregens Factor are still unsettled, but coprogen contains $N^5$-hydroxyornithine units and is therefore in some sense analogous to the ferrichrome group. *Arthrobacter simplex* has been shown to produce ferrioxamine B (53), and it is therefore possible that Terregens Factor derived from *Arthrobacter pascens* is similar. More recently the compounds fusarinine B (68) and fusigen (25) have been described. These have structural features (Fig. 8) resembling ferrirhodin (103), a member of the ferrichrome group, but are also analogous to the ferrioxamine type in having their hydroxamic acid groups as part of a chain rather than as pendant groups in a cyclic peptide structure. In terms of chemical structure the mycobactins are very different from the recognized sideramines. They contain only two hydroxamic acid groups per molecule, though there is a third iron-binding center which apparently stands in place of the third hydroxamic acid. The fragments forming the hydroxamic acid groups in the mycobactins give on hydrolysis $N^6$-hydroxylysine which is obviously a close analogue of $N^5$-hydroxyornithine and of N-hydroxycadaverine which are the respective hydrolysis products of the hydroxamic acids in ferrichrome and ferrioxamine B. An iron-chelating compound, aerobactin (Fig. 9), has recently been isolated from *Aerobacter aerogenes* (30). It resembles the mycobactins in having two hydroxamic acid groups derived from $N^6$-hydroxylysine. The status of aerobactin as a growth factor is not yet established. It may form a hexadentate ferric complex in which the last two coordination positions are occupied by the carboxylic acid group and the hydroxyl group on the central carbon atom of the citric acid unit. Studies with models show such a complex to be feasible. The mycobactins resemble the sideramines in their chelating specificity for ferric iron and in the stability of the ferric complexes (73). Production of the sideramines by microorganisms is greatly stimulated by a deficiency of iron in the growth medium and a similar stimulation is found with mycobactins produced by mycobacteria. The water-soluble sideramines occur widely among microorganisms but have not been reported in mycobacteria. There is thus a prima facie case for regarding the mycobactins as a special type of sideramine

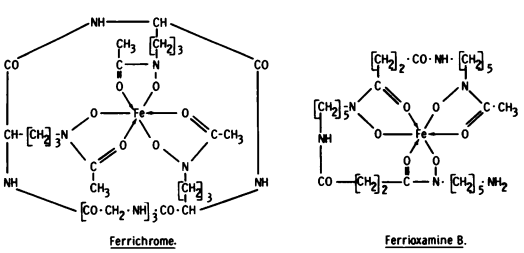



FIG. 7. *Structures of ferrichrome and ferrioxamine B.*

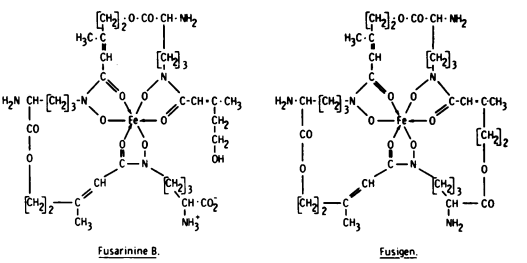



FIG. 8. *Structures of fusarinine B and fusigen.*

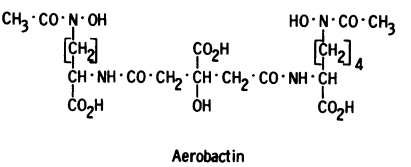



FIG. 9. *Structure of aerobactin.*

peculiar to the mycobacteria. The biochemistry of the water-soluble sideramines will therefore be discussed before returning to the question of the biochemical function of the mycobactins.

## Evidence Concerning the Biochemical Function of Water-Soluble Sideramines

**Iron transport theories.** In the history of the sideramines, the most favored view has been that they are involved in the transport of iron into the cell and in the metabolism of iron within the cell (54). This opinion is based on the following considerations. (i) The exceptionally high affinity and selectivity of the sideramines for ferric iron. (ii) The observed increase of sideramine production in microorganisms in response to iron deprivation. Under these same conditions the same organisms sometimes also secrete porphyrins, precursors of heme, into the medium (29). (iii) The ability of hemin at high concentrations to replace the sideramines in the growth of sideramine-dependent organisms (17, 101). (iv) The apparent universality of sideramines in microorganisms and also possibly in plants (57), in which a mechanism for collecting iron under unfavorable conditions would have a clear biological advantage. Sideramines do not appear to occur in animal cells, in which other means of supplying iron prevail.

The function of the sideramines has been conceived as involving the following sequence of events (101). (i) The production by the cell of the desferrisideramine, in amounts governed by the iron deficit. (ii) Extracellular reaction of the iron with the desferrisideramine; the high affinity will favor a complete saturation of the chelator with iron even at low ionic concentrations. (iii) Transfer of the sideramine into the cell. (iv) Presentation of the sideramine to an enzyme concerned in iron metabolism. (v) Electronic transfer, converting the iron to the ferrous form with release of the desferrisideramine which has little affinity for $Fe^{II}$. (vi) Utilization of the ferrous iron for production of heme, etc., and transfer of the desferrisideramine to the exterior of the cell to gather more iron.

On this basis, the response of the cell to a deficiency of iron by increasing the output of desferrisideramine would tend to increase its efficiency in gathering what little iron was available. At the same time, porphyrin precursors might accumulate through partial blockage at the iron-incorporation step. Hemin overcomes the requirement for sideramine by providing a direct source for the biosynthesis of heme enzymes.

A direct attempt was made by Burnham (15–17) to demonstrate the iron-transporting function of ferrichrome in two different organisms. In the presence of a cell-free extract of *Rhodopseudomonas spheroides*, ferrichrome caused the production of hemin from protoporphyrin IX. The heme was measured by a nitrate reductase method and the transfer of iron from ferrichrome to hemin was shown by use of $^{59}Fe$. This cannot be considered conclusive, however, since ferrichrome, like other sideramines, is able to exchange its iron fairly rapidly with unbound iron and with iron chelated to other compounds such as citrate. Thus, it is by no means certain that ferrichrome was a direct donor of iron in this experiment. Burnham also investigated the action of ferrichrome and ferrimycin A on catalase production by the sideramine-requiring organism *Arthrobacter* JG-9. Addition of hemin allowed the accumulation of catalase in the cells over a 2-hr period and this production was unaffected by the further addition of ferrimycin. Catalase production proceeded similarly after addition of ferrichrome, but in this case ferrimycin greatly reduced the amount of catalase formed. The result was interpreted as showing that ferrimycin functions as an antibiotic by antagonizing the participation of ferrichrome in the synthesis of heme. This experiment has been criticized by Nüesch and Knüsel (56) on the grounds that hemin is unable to counteract the growth-inhibitory effect of ferrimycin and thus the anti-antibiotic action must lie in some other direction.

**Sideromycins.** Any study of the biochemical function of the sideramines requires consideration of the action of the closely related sideromycins. The sideromycins are iron-chelating antibiotics produced by streptomycetes. Two have been investigated in detail; these are albomycin (83, 84) and ferrimycin $A_1$ (11; Fig. 10). The structural relationship of albomycin to ferrichrome and ferrimycin to ferrioxamine B is very striking. Both the sideromycins differ essentially from the corresponding sideramine only in the possession of a complex pendant group. The additional groups present in ferrimycin $A_1$ and in albomycin $\delta_2$ are chemically unrelated. Both are centers of chemical instability, and in

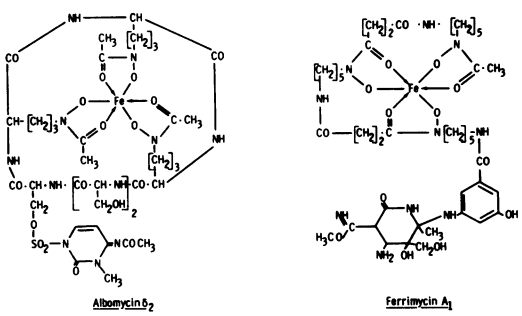



FIG. 10. *Structures of albomycin $\delta_2$ and ferrimycin $A_1$.*

both cases chemical breakdown leads to loss of antibacterial function and to the generation of growth-promoting action similar to that of the corresponding sideramine. This kind of relationship between growth factors and growth antagonists is not readily paralleled elsewhere among natural or synthetic products. In the classic type of metabolic antagonism, the antagonist has a structure sufficiently similar to a substrate or cofactor to occupy an active site on an enzyme or receptor and thus inhibit its action; it must, however, be essentially different in structure so that it cannot participate in the normal function. The sideromycins are peculiar in containing the whole of the structure necessary for sideramine function, and they owe their antagonistic action solely to an extra attachment to the molecule. This feature needs special consideration in trying to decide their mode of action and how this action is related to that of the sideramines.

The relationship between the sideramines and the sideromycins was first explored and defined by Bickel and his colleagues (9), and the study was extended by Zähner et al. (101, 102). These workers demonstrated that the growth-inhibitory action of the ferrimycins towards gram-positive organisms such as *B. subtilis* was competitively relieved by the ferrioxamines, and the growth-inhibitory action of albomycin towards the gram-negative *E. coli* was competitively relieved by sideramines of the ferrichrome type; the ferrioxamines did not antagonize the action of albomycin. It is noteworthy that these experiments were conducted with bacteria that had not previously been known to produce sideramines. The suggestion was made that endogenous sideramines were in fact essential to their growth. The antibacterial action of sideromycins would then involve an antagonism of the normal sideramines of the cell and could be overcome by sideramines supplied exogenously. Sideramines had previously been found mainly in actinomycetes and fungi. There is, however, increasing evidence of the occurrence of the previously recognized sideramines or of other iron-chelating hydroxamic acids in a wide range of bacteria (19, 33, 49, 53, 55); the suggestion that they are universal in aerobic microorganisms may well be true.

The mutual antagonism of the sideromycins and the sideramines in a wide range of bacteria led to the view that the sideromycins owed their antibacterial action to the antagonism of the biochemical function of the sideramines which was envisaged as the presentation of iron to the heme-forming enzymes (101, 102). However, more recent work by Knüsel and his colleagues (41–44, 56, 105) suggested a different interpretation of the previous observations. It now seems more likely that the competitive antagonism of the sideramines towards the antibacterial action of the sideromycins depends upon prevention of their access to the cell. This implies the existence of a specific mechanism by which the sideromycins can be transported into the cell and the blockage of this mechanism by the sideramines; the same mechanism is also blocked in cells that have become resistant to the sideromycins. Direct evidence has been presented (105) of the entry of the sideromycin A 22,765 (possibly identical to danomycin) into cells of *Staphylococcus aureus* and of the blocking of this entry by ferrioxamine B. The mechanism carrying the sideromycins into the cell is probably the one normally used for the entry of the sideramines. The observation that ferrioxamine B is taken up by *S. aureus* to give concentrations only one-twentieth of those attained by A 22,765 is somewhat surprising. However ferrioxamine B is probably not the natural sideramine for *S. aureus* and the sideramine made by this organism may behave differently. The original hypothesis that the sideromycins interfere with the enzymic process of addition of iron to protoporphyrin IX to form heme now seems unlikely (41–43, 56), but the biochemical site of the antibacterial action has not been identified. There is no reason to postulate a connection with the intracellular action of the sideramines. More probably, the structural resemblance of part of the sideromycin molecule to a sideramine serves the purpose of getting the molecule into the cell, and inside the cell the inhibitory action depends entirely on the pendant group. It is then possible to understand the dissimilarity between the pendant groups in ferrimycin $A_1$ and albomycin since the antibacterial action of these two compounds need not involve the same biochemical reaction. This view of the mechanism of sideromycin action may be compared with the chemotherapeutic concept of the "toxophoric group." This is a group having toxic or inhibitory properties that is attached by chemical synthesis to a normal metabolite or growth factor to enable it to enter the cell or to reach a specific site of action; the toxophoric group should then be able to exert its inhibitory action exactly where it is needed. For instance, attempts have been made to obtain specific concentrations of tumor inhibitors within malignant cells by using this principle. There are few analogies to this situation in natural products, but experiments with the antibiotic azaserine have shown a rather similar effect. Azaserine is apparently carried into *E. coli* cells by a permease which normally transports aromatic amino acids. Mutants of *E. coli* were obtained which required tryptophan for growth

but would also grow on indole. Tryptophan requires a permease to enter the cell but indole enters by diffusion. When these mutants were grown with tryptophan in the medium they were insensitive to azaserine, but when grown with indole they were sensitive (39). The tryptophan apparently occupied the permease, denying access to the azaserine, but when indole was substituted the permease was free and azaserine could enter. The action of azaserine within the cell is mainly an interference with the biosynthesis of purines and is apparently not related to the metabolism of the aromatic amino acids.

**Sideramines and aromatic hydroxy acids in bacterial iron uptake.** It is difficult to resist the conclusion that sideramines are involved in the uptake of iron into the cell. However, their action is by no means specific. Sideramines of different structures are interchangeable as growth factors for dependent organisms. The growth of *A. terregens* is stimulated by mycobactin P and less efficiently by cobactin and mycobactic acid, its degradation products (3). Growth stimulation can also be brought about by synthetic fat-soluble chelating agents such as 8-hydroxyquinoline and salicylaldehyde (51). Sideramine growth-promoting action is not confined to trihydroxamic acids among natural products, but is also found with aspergillic acid (17), a dihydroxamic acid, and with schizokinen (19) which is thought to be a monohydroxamic acid.

The stimulating effect of iron deficiency on the production of sideramines and mycobactins has already been discussed. Iron deficiency also stimulates the synthesis and release of various aromatic hydroxy acids. These include 2,3-dihydroxybenzoic acid (18, 98, 99) from *B. subtilis*, *E. coli* and *Aerobacter aerogenes*, 2,3-dihydroxybenzoyl glycine (37, 59) from *B. subtilis*, 2,3-dihydroxybenzoyl serine from *E. coli* (12), $N^2$,$N^6$-di(2,3-dihydroxybenzoyl)-L-lysine (24) from *Azotobacter vinelandii*, 3,4-dihydroxybenzoic acid (5, 20) from *B. anthracis* and *B. cereus*, salicylic acid from *M. tuberculosis* and *M. smegmatis* (63), and 6-methylsalicylic acid from *M. phlei* (64). Many of these chelate iron readily in aqueous solution, though the chelates have stability constants far lower than those of the sideramines. Compounds in which the aromatic hydroxy acid is conjugated to an amino acid may have a special significance in bacterial iron metabolism. In an *E. coli* mutant, the production of the highly specific synthetase that links 2,3-dihydroxybenzoic acid with L-serine in the presence of adenosine triphosphate is strongly repressed by iron, the presence of 1.5 $\mu$M iron in the medium being sufficient to abolish measurable activity (12).

The relationship between the aromatic hydroxy acids and sideramines is still unsettled. Iron deficiency stimulates the production of both, though with iron-deficient *B. subtilis* the excretion of 2,3-dihydroxybenzoic acid was found to be inhibited by 0.2 $\mu$g of desferrischizokinen per ml (18). The gathering of iron from the environment may be divided into two aspects: the solubilization of ferric iron which will often occur in an insoluble and unavailable form, and the conversion of the solubilized iron into a form suitable for absorption by the cell. The function of the aromatic hydroxy acids may perhaps be mainly one of mobilizing the insoluble ferric iron. Wang and Newton (89) found an *E. coli* mutant that failed to produce 2,3-dihydroxybenzoyl serine. This organism grew on media of low iron content only when it was supplied with 0.25 mM 2,3-dihydroxybenzoyl serine or with 10 mM citrate. The function of both compounds was thought to be solubilization of ferric iron, though the former was evidently the more efficient. Iron solubilized as the chelate of one of these aromatic hydroxy acids would be readily transferred to a desferrisideramine because of its much higher affinity for the latter. However, one must question why the iron should not be solubilized and chelated directly by the sideramines. Several explanations are possible, though none has yet been tested experimentally. The aromatic hydroxy acids may be produced in larger quantities and can therefore hold more iron in the chelated form; their acidity may also assist the process of mobilizing the iron (the sideramines being usually neutral in reaction). Kinetic factors must also be considered. Although the sideramines have exceptionally high stability constants, the rate of reaction of the desferri compounds with ferric iron can be relatively slow, especially when both reactants are present in low concentration. The importance of steric factors in the rates of transfer of iron between transferrin and iron chelates has been pointed out (6, 7); strikingly different rates of transfer were observed with citrate, nitrilotriacetate, and ethylenediaminetetraacetate, uncorrelated with the stability constants of their ferric complexes. Similar considerations may apply in other iron-transfer reactions. Thus, the aromatic hydroxy acids and sideramines may be linked in a highly efficient cooperative system for gathering iron.

Whatever the means of collecting iron from the environment, the metal apparently enters the cell in a chelated form through an active mechanism that concentrates iron within the cell. The involvement of an energy-dependent process for iron incorporation has been shown with *B. subtilis* (58) and with *E. coli* (89) in which a 200-

fold concentration of iron within the cells was demonstrated in a medium containing citrate and 6.4 ng of iron per ml. This may imply a permease type of mechanism involving a sideramine. The experiments with the sideromycin A 22,765 (105), discussed above, would support this view. It is not certain whether this process involves active transfer of the sideramine across the membrane. The possibility must be considered that the postulated mechanism of iron absorption is an emergency mechanism operating under conditions of iron deprivation and that bacteria have other means of taking in iron under less exacting conditions. For example, in the experiments of Wang and Newton (88, 89), the *E. coli* mutants which lacked an active iron-transport system were able to grow on media containing a sufficiently high concentration of iron. Further evidence for the existence of a special transport system for the siderochromes is provided by recent experiments on a mutant of *Salmonella typhimurium* blocked in the biosynthesis of 2,3-dihydroxybenzoyl serine (J. R. Pollack et al., Fed. Proc., *in press*). This organism utilized ferrichrome for growth; however, some albomycin-resistant strains derived from it were unable to take up tritium-labeled ferrichrome and could not use it as a growth factor.

### Role of Mycobactins in Mycobacteria

The analogy of the mycobactins to the sideramines has already been noted. The Swiss workers (9, 103) have not regarded the mycobactins as sideramines because they are dihydroxamic acids and because they do not competitively inhibit the antibacterial action of the sideromycins. However, the mycobactins have an iron-chelating ability at least equal to that of the sideramines. The inhibition of sideromycin action probably depends on the structural relationship between the particular sideromycin and the sideramine used, since albomycin is antagonized by the ferrichromes and not by the ferrioxamines. It is not surprising, therefore, that the actions of albomycin and ferrimycin are not antagonized by the mycobactins. Whether the mycobactins are called sideramines is merely a matter of convenience. The main question is whether they are analogous in function. The mycobactins are evidently far more specific than the water-soluble sideramines. They are not known to occur outside the genus *Mycobacterium* and no other type of sideramine or iron-chelating agent has yet been found that will promote the growth of mycobactin-dependent strains of *M. paratuberculosis*. Even the primary breakdown products of mycobactin, cobactin, and mycobactic acid fail either singly or together to stimulate growth of *M. paratuberculosis* (27). It is still uncertain whether the specificity of the mycobactins lies in a fairly narrow structural requirement or whether it is related to the solubility properties which so clearly distinguish the mycobactins from other natural iron chelators so far described.

The mycobacteria show parallel behavior to other bacteria in regard to iron metabolism (61). They respond to iron deficiency by producing an aromatic hydroxy acid, either salicylic acid or 6-methyl-salicylic acid (63, 64), together with compounds of very great affinity for ferric iron, the mycobactins. It seems likely that the considerations already discussed in relation to other bacteria also apply to the mycobacteria, with the mycobactins taking the place of the sideramines. Certain distinctions should be considered, however. With the mycobacteria the aromatic acids are apparently precursors of the mycobactins, whereas with other bacteria there is no biosynthetic connection between the aromatic hydroxy acids and the sideramines. Further, the mycobactins have a low solubility in water and are normally isolated from the cells rather than from the medium. Their situation within the cell is unknown. They may be liberated into the medium at concentrations up to saturation (5 to 10 $\mu$g/ml) and even at this level they readily chelate ferric iron. However, it seems more likely that their function lies within the cell or its envelope. This would further suggest that in the mycobacteria salicylic acid or 6-methyl salicylic acid mobilizes the iron in the environment and that the mycobactins are concerned with the active transport of iron into the cell. The mycobacteria are not very convenient organisms for studying the details of iron uptake and utilization, but the discovery of the competitive inhibition of mycobactin P by chromic mycobactin P in the growth of *M. paratuberculosis* at least provides a new point of attack. Since the chromic complex, unlike other metal complexes of mycobactin, does not exchange its metal at an appreciable rate, its antagonism probably involves the interaction of the whole molecule at some site otherwise occupied by ferric mycobactin. (In growth promotion experiments, no distinction is made between mycobactin and its ferric complex, since at the low concentrations concerned the mycobactin inevitably picks up traces of iron from the medium; mycobactin is preferred for addition to media because of its somewhat greater solubility in aqueous solutions.) The reason for the failure of chromic mycobactin P to antagonize the growth of mycobacteria other than *M. paratuberculosis* (74) is uncertain. These mycobacteria can make their own mycobactins, and one possi-

bility is that they respond to the effect of the chromic complex in the same way as they do to iron deficiency—by making greater quantities of mycobactin. This would overcome the inhibitory action. Work with chromic mycobactin using cell-free extracts of mycobacteria may help to clear up some of the present uncertainties of mycobactin action.

## SUMMARY AND CONCLUSIONS

The mycobactins are a group of bacterial growth factors that present many features of interest. Historically, they have occupied a rather neglected side line of microbiological and biochemical research, but they have on several occasions provided novel observations that have later been shown to have a more general significance. Their discovery, reported in 1912, probably represented the first clear recognition of a specific bacterial growth factor. Mycobactin P, isolated in 1946, was the first example of a now widening group of natural products that are distinguished by exceptional iron-chelating activity; it was also the first of these to be structurally characterized. The hydrolytic degradation of mycobactin P gave a new type of amino acid, shown to be $N^6$-hydroxylysine; similar hydroxyamino compounds were later found to be characteristic hydrolysis products from other natural iron-chelating compounds. Among the many bacterial growth factors with iron-binding properties now known, the mycobactins retain a unique position since their occurrence is apparently confined to the bacterial genus *Mycobacterium*; within this genus they exert a well-marked growth-promoting action which is not shared by any other known type of compound. The growth factors from different mycobacteria were originally supposed to be chemically identical, but at least nine mycobactins are now known to exist; all possess a similar basic molecular pattern but are distinguished by variations in details of structure. Adansonian methods have been used to divide the mycobacteria into groups or clusters of related species; when representative organisms from each group were examined, each yielded a chemically distinct type of mycobactin. The relationship between classification of the species and the chemical structure of the mycobactins may in future provide a useful taxonomic tool.

The potential interest of the mycobactins is by no means exhausted. The problem of their biochemical function still remains. Circumstantial evidence suggests that they are concerned with iron transport, but they may also have some other more specific action within the cell. This problem is linked to that of the action of the water-soluble sideramines, but the higher degree of specificity of the mycobactins may permit a more decisive answer to be reached. The mycobactins also present problems in biosynthesis that have so far received only slight attention. Among the component parts of the mycobactin molecule the source of the aromatic components, whether from acetate or shikimate, is still to be fully worked out; the mechanism of N-hydroxylation also requires further clarification. The mycobactins represent one of the best examples of natural compounds in which component parts are derived from different biosynthetic pathways. The order in which these parts are assembled and the mechanisms that control the synthesis of the components in appropriate quantities are fascinating subjects for further research.

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